Seawater Polluted with Highly Concentrated Polycyclic Aromatic Hydrocarbons Suppresses Osteoblastic Activity in the Scales of Goldfish, Carassius auratus

著者	Suzuki Nobuo, Sato Masayuki, Nassar Hossam F., Abdel-Gawad Fagr Kh., Bassem Samah M., Yachiguchi Koji, Tabuchi Yoshiaki, Endo Masato, Sekiguchi Toshio, Urata Makoto, Hattori Atsuhiko, Mishima Hiroyuki, Shimasaki Youhei, Oshima Yuji, Hong Chun-Sang, Makino Fumiya, Tang Ning, Toriba Akira, Hayakawa Kazuichi
journal or publication title	Zoological Science
volume	33
number	4
page range	407-413
year	2016-08-01
URL	http://hdl.handle.net/2297/46166

doi: 10.2108/zs150211

Physiology

Seawater polluted with highly concentrated polycyclic aromatic hydrocarbons suppresses osteoblastic activity in the scales of goldfish, *Carassius auratus*

Nobuo SUZUKI[a*], Masayuki SATO[a], Hossam F. NASSAR[b], Fagr Kh. ABDEL-GAWAD[b], Samah M. BASSEM [b], Koji YACHIGUCHI[a], Yoshiaki TABUCHI[c], Masato ENDO[d], Toshio SEKIGUCHI[a], Makoto URATA[a,e], Atsuhiko HATTORI[f], Hiroyuki MISHIMA[g], Youhei SHIMASAKI[h], Yuji OSHIMA[h], Chun-Sang HONG[i], Fumiya MAKINO[j], Ning TANG[j], Akira TORIBA[j] and Kazuichi HAYAKAWA[a,j]

[a]Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University, Housu-gun, Ishikawa 927-0553, Japan
[b]Environmental Research Division, Water Pollution Control Department, National Research Center, Cairo 12621, Egypt.
[c]Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Sugitani, Toyama 930-0194, Japan
[d]Department of Marine Biosciences, Division of Marine Science, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan
[e]Institute of Noto SATOUMI Education Research, Noto-cho, Ishikawa 927-0553, Japan
[f]Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272-0827, Japan
[g]Department of Medical Hygiene, Kochi Gakuen College, Kochi 780-0955, Japan
[h]Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan
[i]Hankuk University of Foreign Studies, 81, Oedae-ro, Mohyeon-myeon, Cheoin-gu, Yongin-si, Gyeonggi-do 17035, Korea
[j]Faculty of Pharmaceutical Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma, Ishikawa 920-1192, Japan

Abbreviated title: PAH polluted-water inhibits osteoblasts

*Correspondence to: Dr. N. Suzuki, Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University, Ogi, Noto-cho, Ishikawa 927-0553, Japan. Tel.: 81-768-74-1151; Fax 81-768-74-1644; e-mail: nobuos@staff.kanazawa-u.ac.jp

ABSTRACT

We have developed an original in vitro bioassay using teleost scale that has osteoclasts, osteoblasts, and bone matrix as each marker: alkaline phosphatase (ALP) for osteoblasts and tartrate-resistant acid phosphatase (TRAP) for osteoclasts. Using this scale in vitro bioassay, we examined the effects of seawater polluted with highly concentrated polycyclic aromatic hydrocarbons (PAHs) and nitro-polycyclic aromatic hydrocarbons (NPAHs) on osteoblastic and osteoclastic activities in the present study. Polluted seawater was collected from 2 sites (the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea). Total levels of PAHs in the seawater from the Alexandria and Suez Canal sites were 1364.59 and 992.56 ng/l, respectively. We can detect NPAHs in both seawater samples. Total levels of NPAHs were detected in the seawater of the Alexandria site (12.749 ng/l) and the Suez Canal site (3.914 ng/l). Each sample of polluted seawater was added into culture medium at dilution rates of 50, 100, and 500 and incubated with the goldfish scales for 6 hrs. Thereafter, ALP and TRAP activities were measured. As a result, ALP activity was significantly suppressed by both polluted seawater samples diluted at least 500 times, although TRAP activity did not change. In addition, mRNA expressions of osteoblastic markers (ALP, osteocalcin, and the receptor activator of the NF-κB ligand) decreased significantly, as did the ALP enzyme activity. Actually, ALP activity decreased with selected PAHs and NPAHs treatments. We conclude that seawater polluted with highly concentrated PAHs and NPAHs influenced bone metabolism in teleosts.

Keywords: polycyclic aromatic hydrocarbons; nitro-polycyclic aromatic hydrocarbons; osteoblasts; osteoclasts; fish scales; bone metabolism; goldfish; bioassay

INTRODUCTION

Oil spills on the sea surface are seen relatively often. Observed oil spills correlate very well with the major shipping routes (Brekke and Solberg, 2005). In addition, accidental oil spills have occurred. Big oil spills from the Deepwater Horizon and sunken oil tanker ships (the Exxon Valdez and the Nakhodka) directly caused oil pollution in the marine environment and affected marine organisms (Bue et al., 1998; Heintz et al., 2000; Hayakawa et al., 2006; de Soysa et al., 2012). Oil contamination can seriously impact the marine environment toxicologically. Polycyclic aromatic hydrocarbons (PAHs) are toxic substances in oil (Hayakawa et al., 2006). For a long time (more than 14 years) after big oil spill from the Exxon Valdez, the toxicity of PAHs originating from spilled oil has been observed in numerous marine animals (Peterson, 2003). In fish, which are representative of aquatic animals, PAHs have been reported to cause bone deformities in Pacific herring, pink salmon, and sea bass (Barron et al., 2004; Danion et al., 2011). Therefore, more attention should be given to the metabolism of fish bone.

The teleost scale is calcified tissue that contains osteoblasts, osteoclasts, and the two layers of bone matrix, i.e., a bony layer, which is a thin, well-calcified external layer, and a fibrillary layer, which is a thick, partially calcified layer (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007; Azuma et al., 2007; Ohira et al., 2007). The bone matrix, which includes type I collagen (Zylberberg et al., 1992), osteocalcin (Nishimoto et al., 1992), and hydroxyapatite (Onozato et al., 1979), is present in scale as well as in mammalian bone. Furthermore, it is known that teleost scale regenerates after being removed. We previously reported that the osteogenesis in regenerating scales was very similar to that in calvarial bone (mammalian head bone) (Yoshikubo et al., 2005).

Osteoblastic activity in the regenerating scale was considerably higher than that in normal scale (Yoshikubo et al., 2005; Suzuki et al., 2009). The response of osteoblasts to estrogen was higher in regenerating scales than in normal scales (Yoshikubo et al., 2005). In regenerating scales, osteoclastic activity was at the same level as osteoblastic activity and sensitively responded to gravity loading (Suzuki et al., 2009). These facts indicate that regenerating scale can be utilized as a model for teleost bone to analyze the influence of PAHs on osteoblasts and osteoclasts.

For the purpose of analyzing marine pollution from oil spills, in the present study, we used our scale in vitro bioassay system to examine the influence on osteoblasts and osteoclasts of polluted seawater samples from the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea.

MATERIALS AND METHODS

Animals

Goldfish (*Carassius auratus*), purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan), were artificially fertilized from female and male goldfish (20–30 g) in the Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology. Fish were fed a commercial pellet diet for puffer fish every morning and were maintained in fresh water at 26°C. Growing fish 12–15 cm in body length were moved to Noto Marine Laboratory in Kanazawa University and used for in vitro experiments analyzing cell activity and mRNA expression.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

Sampling sites and analysis of PAHs and nitro-polycyclic aromatic hydrocarbons (NPAHs) in seawater samples

Surface water was collected from the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea (Figure 1). There are very many shipping lanes in these sea areas. The sampling, extraction, and analysis of PAHs and nitro-polycyclic aromatic hydrocarbons (NPAHs) were performed as follows.

Water samples were collected using narrow-neck glass 2-liter bottles (previously washed with dichloromethane). One hundred ml (5%) of methanol was added to each sample. The sample was filtered by using a glass fiber filter (GC-50, 0.45-μm poor size, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and then the filtrate was filtered again by using a 3M Empore solid phase extraction disk (C18, ϕ47 mm, 3M Company, Saint Paul, MN, USA). The 3M disks were ultrasonically twice extracted with dichloromethane and then the solution was filtered through a glass filter paper. After addition of 100-μl dimethyl sulfoxide (DMSO), the filtrate was concentrated. The residue containing 100-μl DMSO was dissolved in 900-μl of ethanol. The final sample solution was filtered through a membrane filter (HLC-DISK13, 0.45-μm poor size, Kanto Chemical Co., Inc., Tokyo, Japan). Other conditions were the same as in our previous reports (Tang et al., 2005a, 2005b; Hattori et al., 2007; Nassar et al., 2011).

We analyzed fifteen PAHs using HPLC with fluorescence detection. The system consisted of two HPLC pumps (LC-10A, Shimadzu, Kyoto, Japan), a fluorescence detector (RF-10A,

Shimadzu), a system controller (SCL-10A, Shimadzu), an integrator (Chromatopac C-R7Ae, Shimadzu), a degasser (DGU-14A, Shimadzu), an auto sample injector (SIL-10A, Shimadzu), a column oven (CTO-10AS, Shimadzu), a guard column (Inertsil ODS-P, 4.0 i.d. × 10 mm, GL Sciences Inc., Tokyo, Japan), and an analytical column (Inertsil ODS-P, 4.6 i.d. × 250 mm, GL Sciences Inc., Tokyo, Japan). The mobile phase was a mixture of acetonitrile and water with a gradient concentration mode of acetonitrile. The flow rate was 1.0 ml/min. The fluorescence time program detector was set to detect at the optimum excitation and emission wavelength for each PAH.

NPAHs were analyzed by HPLC with chemiluminescence detection with several modifications in accordance with our laboratory detection method (Tang et al., 2009; Nassar et al., 2012, 2015). The HPLC system consisted of two analytical columns (both Cosmosil 5C18-MS, 4.6 i.d. × 10 mm, Nacalai Tesque, Kyoto, Japan), two mobile phase pumps (LC-10A, Shimadzu, Kyoto, Japan), a chemiluminescence reagent solution pump (DMX-2000, Sanuki, Tokyo, Japan), a chemiluminescence detector (CLD-10A, Shimadzu, Kyoto, Japan), a system controller (SCL-10A, Shimadzu, Kyoto, Japan), a chromatopac integrator (C-R4A, Shimadzu, Kyoto, Japan), a degasser (DGU-14A, Shimadzu, Kyoto, Japan), an auto sample injector (SIL-10A, Shimadzu, Kyoto, Japan), a column oven (CTO-10AC, Shimadzu, Kyoto, Japan), and a guard column (Cosmosil 5C18-MS, 4.6 i.d. × 10 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was imidazole–perchloric acid buffer (pH 7.6) : acetonitrile (1:1) at a flow rate of 1.0 ml/min. The post-column chemiluminescence reagent solution was an acetonitrile solution containing 0.02 mM *bis*(2,4,6-trichlorophenyl) oxalate and 15 mM hydrogen peroxide at a flow rate of 1.0 ml/min.

The validity of the above-mentioned method was already confirmed in our previously published papers, which illustrated recovery variations of 87 to 104%; limits of detection

(S/N = 3) varied from 0.25 to 1.5 x 10^{-15} mol, and limits of quantification (S/N = 10) varied from 10^{-15} to 10^{-12} mol (over two orders) and showed good linearity ($r^2 \geq 0.899$) (Tang et al., 2005a; Hattori et al., 2007; Nassar et al., 2011).

Effects of polluted-seawater samples on scale osteoblastic and osteoclastic activities using cultured scales of goldfish

Goldfish were anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., St. Louis, MO, USA), and the normally developed scales on the body were removed to allow the regeneration of scales. On day 14, goldfish were anesthetized again, and the regenerating scales were removed. Using the removed regenerating scales, we examined the influences of seawater samples on the osteoblasts and osteoclasts with alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) as markers because, in mammals, the effects of hormones and some bioactive substances on osteoblasts and osteoclasts have been investigated using ALP and TRAP as respective markers (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999). These scales were incubated for 6 hrs in Leibovitz's L-15 medium (Phenol Red-free, Invitrogen, Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., Aurora, OH, USA) supplemented with polluted seawater (diluted 50, 100, and 500 times with L-15 medium). The influences of polluted seawater on osteoblasts and osteoclasts were compared with that of artificial seawater (Allen seawater: NaCl 3%, $MgSO_4 \cdot 7H_2O$ 0.358%, $MgCl_2 \cdot 6H_2O$ 0.272%, $CaCl_2 \cdot 2H_2O$ 0.06%, KCl 0.039%, $NaHCO_3$ 0.01%; Suzuki et al., 1992) as a non-polluted seawater.

From one goldfish, 24 scales from the left or right side were utilized. The 24 scales used in the present study were used as follows: (1) 8 scales for ALP analysis with 500-times dilution, (2) 8 scales for ALP analysis with 100-times dilution, (3) 8 scales for ALP analysis with 50-times dilution. The respective mean for ALP (obtained from 8 individual scales of one goldfish) was compared with that of the right side (control group: diluted artificial seawater samples). After incubation, ALP activities were measured using the same methods previously described (Suzuki et al., 2009). The results are shown as the means ± SEM (n = 10). In the case of TRAP, the same experiment was done repeatedly using 10 individual goldfish.

The methods for measuring ALP and TRAP activities were as follows. An aliquot of 100 µl of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$) or an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) was added to each well. Then, each scale was put into its own well in a 96-well microplate. This microplate was immediately frozen at -80°C and then kept at -20°C until analysis. An aliquot of 100 µl of 20 mM para-nitrophenyl phosphate in an acid or alkaline buffer was then added to each well of melted solution in the microplate. This plate was incubated at 23°C for 20 min, while being shaken. After incubation, the reaction was stopped by adding 50 µl of 3 N NaOH. One hundred fifty µl of a colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of para-nitrophenol (pNP) produced using a standard curve for pNP.

After measuring both ALP and TRAP activities, the scales were measured with Image J. Afterward, ALP and TRAP activities were normalized to the surface area (mm^2) of each scale (Suzuki et al., 2009).

Changes in osteoblastic and osteoclastic marker mRNA expressions in polluted seawater-treated goldfish scales

Scales were collected from goldfish anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich). To examine changes in osteoblastic (ALP, osteocalcin, and the receptor activator of nuclear factor κB ligand: RANKL) and osteoclastic (cathepsin K, vacuole-ATPase, and matrix metalloproteinase-9: MMP-9) marker mRNAs that responded to polluted seawater, these scales were incubated for 6 hrs in L-15 medium (Phenol Red-free, Invitrogen) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals) that included polluted seawater or artificial seawater samples (each diluted 50 times). After incubation, the scales were frozen at -80 °C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for ALP (sense: 5'-TGGACACAGCGGTGAGGAAA-3'; antisense: 5'-GTGGGCATATGCTGCACTCG-3') (Thamamongood et al., 2012), osteocalcin (sense: 5'-ATGCCTGAGCGCAGGTCTTC-3'; antisense: 5'-CACAGGCCAGGTTTGCTTCA-3') (Thamamongood et al., 2012), RANKL (sense: 5'- CGAGTGTGGCGATTTTGTTG -3'; antisense: 5'-ATGGGCGTCTTGATTGGAAG-3') (AB894120), cathepsin K (sense: 5'-TGGGAGGGCTGGAAACTCAC-3'; antisense: 5'-CATGAGCCGCATGAACCTTG-3') (AB236969), vacuole-ATPase (sense: 5'-ACACCGCTTGCTGCTTTCTTTC-3'; antisense: 5'-ACCAGTGTGGAGCAGAACTTG-3') (AB894122), and MMP-9 (sense: 5'-

GCTTCTGCCCCAGTGAGCTT-3'; antisense: 5'-GTGGAGCACCAGCGATACCC-3') (AB889498) were used. The amplification of EF1α cDNA using a primer set (sense: 5'-ATTGTTGCTGGTGGTGTTGG-3'; antisense: 5'-GGCACTGACTTCCTTGGTGA-3') (AB979720) was performed. The PCR amplification was analyzed by real-time PCR apparatus (Mx3000p, Agilent Technologies, Santa Clara, CA, USA) (Suzuki et al., 2011a). The annealing temperature of ALP, osteocalcin, RANKL, vacuole-ATPase, cathepsin K, MMP-9, and EF1α was 60°C. The ALP, osteocalcin, RANKL, vacuole-ATPase, cathepsin K, and MMP-9 mRNA levels were normalized to the EF1α mRNA level.

Effects of PAHs and NPAHs on scale osteoblastic activity using cultured scales of goldfish

In both the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea, highly concentrated PAHs (naphthalene and acenaphthene) and NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) were selected. The influence of these chemicals on ALP activity in scales was examined to confirm the toxicity of PAHs and NPAHs on fish bone metabolism. The concentrations of PAHs (naphthalene and acenaphthene) and NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) were 6 ng/l and 40 pg/l, respectively. The concentrations of these chemicals used in this experiment corresponded to approximately 50-times dilutions of polluted seawater, as indicated in Table 1.

The preparation and incubation of regenerating goldfish scales were described above. After 6 hrs of incubation supplemented with PAHs and NPAHs, ALP activity was measured and normalized to the surface area (mm^2) of each scale (Suzuki et al., 2009).

Statistical analysis

All results are expressed as the means ± SE. The statistical significance between the control and experimental groups was assessed by paired *t*-test. In all cases, the selected significance level was $P < 0.05$.

RESULTS

Analysis of PAHs and NPAHs in the Alexandria and Suez Canal sites

Polluted seawater was collected from 2 sites (the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea). The results are indicated in Table 1. In the seawater of the Alexandria and Suez Canal sites, the levels of total PAHs were 1364.59 and 992.56 ng/l, respectively. In addition, we detected NPAHs in both seawater samples. Total levels of NPAHs were detected in the seawater of the Alexandria site (12.749 ng/l) and the Suez Canal site (3.914 ng/l).

Effects of seawater samples on scale osteoblastic and osteoclastic activities using the cultured scales of goldfish

Each polluted seawater sample was added to culture medium diluted 50, 100, or 500 times and incubated with the goldfish scales for 6 hrs. Thereafter, ALP and TRAP activities were measured. The diluted seawater from both sites significantly suppressed ALP activity (500-time dilution: $P < 0.05$; 100-time dilution: $P < 0.05$; 50-time dilution: $P < 0.01$) as compared with diluted artificial seawater (Fig. 2).

On the other hand, polluted seawater from both sites did not influence TRAP activity at least present conditions (Fig. 3).

The influences of polluted seawater in osteoblastic and osteoclastic marker mRNA expressions

Osteoblastic and osteoclastic marker results are indicated in Figs. 4 and 5, respectively.

At 6 hrs of incubation after treatment of polluted seawater from the Alexandria and Suez Canal sites, the mRNA expressions of osteoblastic markers (ALP, osteocalcin, and the receptor activator of the NF-κB ligand: RANKL) significantly ($P < 0.05$) decreased, as did the ALP enzyme activity, although polluted seawater from both sites did not influence the expression of osteoclastic marker (cathepsin K, vacuole-ATPase, and MMP-9) mRNA expression at least present conditions.

Effects of PAHs and NPAHs on scale osteoblastic activity using cultured scales of goldfish

In both the Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea, the high concentrated-PAHs (naphthalene and acenaphthene) and -NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) were selected. The influences of these chemicals on scales ALP activity were examined in the present study.

With the addition of each chemical, ALP activity in the scales of goldfish decreased (Table 2). Furthermore, ALP activity in the scales of goldfish was significantly suppressed with acenaphthene treatment (Table 2).

DISCUSSION

In the present study, we found that polluted seawater from Alexandria site on the Mediterranean Sea and the Suez Canal site on the Red Sea affected the osteoblastic activity of goldfish scales. In addition, osteoblastic markers such as ALP, osteocalcin, and RANKL mRNA expression were suppressed by the polluted seawater. We believe that one of the toxic causative agents is PAH because bone deformity was reportedly caused in fresh water teleosts (zebrafish and Medaka) (Billiard et al., 2006; Farwell et al., 2006) as well as seawater teleosts (Pacific herring, pink salmon, and sea bass) (Barron et al., 2004; Danion et al., 2011) by PAHs.

Benzo[*a*]pyrene (BaP) is a kind of PAH well known as a toxic substance and carcinogen (Kizu et al., 2003; Moffat et al., 2015). Epidemiological and experimental animal studies associate exposure to BaP with an increased risk of several forms of cancer, including tumors in the forestomach, oral cavity, liver, and lung (Moffat et al., 2015). Furthermore, we can detect NPAHs, such as 1-nitropyrene, 3-nitrobenzanthrone, and 6-nitrochrysene, in samples from the Alexandria and Suez Canal sites. It has been reported that 1-nitropyrene, 3-nitrobenzanthrone, and 6-nitrochrysene are toxic compounds (Hayakawa et al., 1997; Mitchell and Thomassen, 1990; Taga et al., 2005; Huang et al., 2014). Also, 1-nitropyrene and 6-nitrochrysene were detected in the bodies of trout collected from Lake Michigan (Huang et al., 2014). In consideration of above-mentioned toxicity of PAHs and NPAHs, we examined the influences of highly concentrated PAHs (naphthalene and acenaphthene) and NPAHs (2-nitrofluorene and 3-nitrobenzanthrone) on the osteoblastic activity of scales. As a result, we found that acenaphthene was toxic to osteoblastic activity in goldfish scales. Furthermore, with the addition of naphthalene, 2-nitrofluorene, and 3-nitrobenzanthrone, osteoblastic

activity tended to decrease. Thus, we concluded that polluted seawater suppressed osteoblastic activity in the scales of goldfish through the additive and/or synergistic actions of these PAHs and NPAHs.

Our in vitro bioassay system can detect the activities of both scale osteoblasts and osteoclasts with TRAP and ALP as markers (Persson et al., 1995; Suzuki et al., 2007; de Vrieze et al., 2010). The enzyme activity in one scale can be detected by transferring it into a 96-well microplate and directly incubating it with the substrate in each well. Using this in vitro system, the effects of endocrine disrupters—such as bisphenol-A (Suzuki and Hattori, 2003), tributyltin (Suzuki et al., 2006), and polychlorinated biphenyl (Yachiguchi et al., 2014a)—and heavy metals (i.e., cadmium and organic mercury) (Suzuki et al., 2004; Suzuki et al., 2011b; Yachiguchi et al., 2014a) on osteoblasts and osteoclasts have been measured. In the case of cadmium, its concentration (even at 10^{-13} M) influenced osteoclastic activity in the scale (Suzuki et al., 2004). Also, even 10^{-10} M tributyltin significantly inhibited osteoblastic activity. Furthermore, polychlorinated biphenyl 118 influenced osteoclastic activity at a concentration of 0.025 ppm (around 10^{-8} to 10^{-7} M) (Yachiguchi et al., 2014b). In the present study, we can sensitively evaluate the effect of seawater polluted by PAHs and NPAHs on osteoblasts for short incubation times. Thus, our bioassay system is very useful for evaluating the effect of environmental pollutants on bone metabolism.

In Sardinia of Western Mediterranean Sea, total concentrations of PAHs ranged from 0.272 to 1.392 ng/l with a mean value of 0.623 ng/l (Marrucci et al., 2013). PAH levels in Sardinia were lower than those in the Japan Sea (8.5 ng/l) (Chizhova et al., 2013). In the seawater of the Alexandria site, however, a high level of PAHs (1364.59 ng/l) was detected. PAHs derived from ship oil are more likely to be a cause of oil pollution because oil spills correlated very well with the major shipping routes in marine environments (Brekke and

Solberg, 2005). Thus, we will examine the toxic influence of PAHs and NPAHs on the bone metabolism of fish in the polluted marine area and call for the prevention of PAH pollution from ship oil.

ACKNOWLEDGMENTS

This study was supported in part by grants to N.S. (Grant-in-Aid for Scientific Research [C] No. 24620004 by JSPS), to Y.T. (Grant-in-Aid for Scientific Research [B] No. 24310046 by JSPS), to A.H. (Grant-in-Aid for Scientific Research [C] No. 24570068 by JSPS), to T.S. (Scientific Research [C] No. 15K07126 by JSPS), and to H.M. (Scientific Research [C] No. 15K11034 by JSPS). This work was performed under the cooperative research program of Institute of Nature and Environmental Technology, Kanazawa University Accept No.5 in 2015.

REFERENCES

Azuma K, Kobayashi M, Nakamura M, Suzuki N, Yashima S, Iwamuro S, Ikegame M, Yamamoto T, Hattori A (2007) Two osteoclastic markers expressed in multinucleate osteoclasts of goldfish scales. Biochem Biophys Res Commun 362: 594-600

Brekke C, Solberg AHS (2005) Oil spill detection by satellite remote sensing. Remote Sens Environ 95: 1 –13

Barron MG, Carls MG, Heintz R, Rice SD (2004) Evaluation of fish early life-stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures. Toxicol Sci 78: 60-67

Bereiter-Hahn J, Zylberberg L (1993) Regeneration of teleost fish scale. Comp Biochem Physiol 105A: 625-641

Billiard SM, Timme-Laragy AR, Wassenberg DM, Cockman C, Di Giulio RT (2006) The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. Toxicol Sci 92: 526-536

Bue BG, Sharr S, Seeb JE (1998) Evidence of damage to pink salmon populations inhabiting prince William Sound, Alaska, two generations after the Exxon Valdez oil spill. Trans Am Fish Soc 127: 35-43

Chizhova T, Hayakawa K, Tishchenko P, Nakase H, Koudryashova Y (2013) Distribution of PAHs in the northwestern part of the Japan Sea. Deep-Sea Res II 86-87: 19-24

Danion M, Deschamps MH, Thomas-Guyon H, Bado-Nilles A, Le Floch S, Quentel C, Sire JY (2011) Effect of an experimental oil spill on vertebral bone tissue quality in European sea bass (*Dicentrarchus labrax L.*). Ecotoxicol Environ Saf 74: 1888-1895

de Soysa TY, Ulrich A, Friedrich T, Pite D, Compton SL, Ok D, Bernardos RL, Downes GB, Hsieh S, Stein R, Lagdameo MC, Halvorsen K, Kesich LR, Barresi MJ (2012) Macondo crude oil from the Deepwater Horizon oil spill disrupts specific developmental processes during zebrafish embryogenesis. BMC Biol 10, 40.

de Vrieze E, Metz JR, Von den Hoff, Flik G (2010) ALP, TRAcP and cathepsin K in elasmoid scales: a role in mineral metabolism. J Appl Ichthyol 26: 210-213

Dimai HP, Linkhart TA, Linkhart SG, Donahue LR, Beamer WG, Rosen CJ, Farley JR, Baylink DJ (1998) Alkaline phosphatase levels and osteoprogenitor cell numbers suggest bone formation may contribute to peak bone density differences between two inbred strains of mice. Bone 22: 211-216

Farwell A, Nero V, Croft M, Bal P, Dixon DG (2006) Modified Japanese medaka embryo-larval bioassay for rapid determination of developmental abnormalities. Arch Environ Contam Toxicol 51: 600-607

Hattori T, Tang N, Tamura K, Hokoda A, Yang X, Igarashi K, Ohno M, Okada Y, Kameda T, Toriba A, Hayakawa K (2007) Particulate polycyclic aromatic hydrocarbons and their nitrated derivatives in three cities in Liaoning Province, China. Environ Forensic 8: 165–172

Hayakawa K, Nakamura A, Terai N, Kizu R, Ando K (1997) Nitroarene concentrations and direct-acting mutagenicity of diesel exhaust particulates fractionated by silica-gel column chromatography. Chem Pharm Bull 45: 1820-1822

Hayakawa K, Nomura M, Nakagawa T, Oguri S, Kawanishi T, Toriba A, Kizu R, Sakaguchi T, Tamiya E (2006) Damage to and recovery of coastlines polluted with C-heavy oil spilled from the Nakhodka. Water Res 40: 981-989

Heintz RA, Rice SD, Wertheimer AC, Bradshaw RF, Thrower FP, Joyce JE, Short JW (2000) Delayed effects on growth and marine survival of pink salmon *Oncorhynchus gorbuscha* after exposure to crude oil during embryonic development. Mar Ecol Prog Ser 208: 205-216

Huang L, Chernyak SM, Batterman SA (2014) PAHs, nitro-PAHs, hopanes, and steranes in lake trout from Lake Michigan. Environ Toxicol Chem 33: 1792–1801

Kizu R, Okamura K, Toriba A, Kakishima H, Mizokami A, Burnstein KL, Hayakawa K (2003) A role of aryl hydrocarbon receptor in the antiandrogenic effects of polycyclic aromatic hydrocarbons in LNCaP human prostate carcinoma cells. Arch Toxicol 77: 335-343

Marrucci A, Marras B, Campisi SS, Schintu M (2013) Using SPMDs to monitor the seawater concentrations of PAHs and PCBs in marine protected areas (Western Mediterranean). Mar Pollut Bull 75: 69-75

Mitchell CE, Thomassen DG (1990) Cytotoxic and transformation responses of rat tracheal epithelial cells exposed to nitrated polycyclic aromatic hydrocarbons in culture. Carcinogenesi 11:155-158

Moffat I, Chepelev N, Labib S, Bourdon-Lacombe J, Kuo B, Buick JK, Lemieux F, Williams A, Halappanavar S, Malik A, Luijten M, Aubrecht J, Hyduke DR, Fornace AJ Jr, Swartz CD, Recio L, Yauk CL (2015) Comparison of toxicogenomics and traditional approaches to inform mode of action and points of departure in human health risk assessment of benzo[a]pyrene in drinking water. Crit Rev Toxicol 45: 1-43

Nassar HF, Tang N, Kameda T, Toriba A, Khoder MI, Hayakawa K (2011) Atmospheric concentrations of polycyclic aromatic hydrocarbons and selected nitrated derivatives in Greater Cairo, Egypt. Atmos Environ 45: 7352- 7359

Nassar HF, Kameda T, Toriba A, Hayakawa K (2012) Characteristics of polycyclic aromatic hydrocarbons and selected nitro derivatives in Cairo, Egypt from the comparison with Japanese typical traffic and industrial cities. In “Proceedings of the 2012 INEF Environmental Forensic Conference” Eds by R Morrison and G O'Sullivan, The Royal Society of Chemistry, Cambridge, UK 2: 171-180

Nassar HF, Tang N, Toriba A, Abdel Gawad F Kh, Hayakawa K (2015) Occurrence and risk assessment of polycyclic cyclic aromatic hydrocarbons (PAHs) and their nitrated derivatives (NPAHs) at Nile river and Esmailia canal in Egypt. Int J Sci Eng Res 8: 1983-2006

Nishimoto NK, Araki N, Robinson FD, Waite JH (1992) Discovery of bone γ-carboxyglutamic acid protein in mineralized scales. J Biol Chem 267:11600-11605

Ohira Y, Shimizu M, Ura K, Takagi Y (2007) Scale regeneration and calcification in goldfish *Carassius auratus*: quantitative and morphological processes. Fisherys Sci 73: 46-54

Onozato H, Watabe N (1979) Studies on fish scale formation and resorption III: Fine structure and calcification of the fibrillary plates of the scales in *Carassius auratus* (Cypriniformes: Cyprinidae). Cell Tissue Res 201: 409-422

Persson P, Takagi Y, Björnsson BTh (1995) Tartrate resistant acid phosphatase as a marker for scale resorption in rainbow trout, *Oncorhynchus mykiss*: Effects of estradiol-17β treatment and refeeding. Fish Physiol Biochem 14: 329-339

Peterson CH, Rice SD, Short JW, Esler D, Bodkin JL, Ballachey BE, Irons DB (2003) Long-term ecosystem response to the Exxon Valdez oil spill. Science 302: 2082-2086

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20: 345-357

Suzuki N, Yazawa K, Watanabe K, Akahori Y, Ishikawa C, Kondo K, Takada K (1992) Culture conditions of Marine bacterium SCRC-2738 for the production of eicosapentaenoic acid (EPA). Nippon Suisan Gakkaishi 58: 323-328

Suzuki N, Suzuki T, Kurokawa T (2000) Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). Peptides 21: 115-124

Suzuki N, Hattori A (2003) Bisphenol A suppresses osteoclastic and osteoblastic activities in the cultured scales of goldfish. Life Sci 73: 2237-2247

Suzuki N, Yamamoto M, Watanabe K, Kambegawa A, Hattori A (2004) Both mercury and cadmium directly influence calcium homeostasis resulting from the suppression of scale bone cells: The scale is a good model for the evaluation of heavy metals in bone metabolism. J Bone Miner Metab 22: 439-446

Suzuki N, Tabata MJ, Kambegawa A, Srivastav AK, Shimada A, Takeda H, Kobayashi M, Wada S, Katsumata T, Hattori A (2006) Tributyltin inhibits osteoblastic activity and disrupts calcium metabolism through an increase in plasma calcium and calcitonin levels in teleosts. Life Sci 78: 2533-2541

Suzuki N, Kitamura K, Nemoto T, Shimizu N, Wada S, Kondo T, Tabata MJ, Sodeyama F, Ijiri K, Hattori A (2007) Effect of vibration on osteoblastic and osteoclastic activities: Analysis of bone metabolism using goldfish scale as a model for bone. Adv Space Res 40: 1711-1721

Suzuki N, Kitamura K, Omori K, Nemoto T, Satoh Y, Tabata MJ, Ikegame M, Yamamoto T, Ijiri K, Furusawa Y, Kondo T, Takasaki I, Tabuchi Y, Wada S, Shimizu N, Sasayama Y, Endo M, Takeuchi T, Nara M, Somei M, Maruyama Y, Hayakawa K, Shimazu T, Shigeto Y, Yano S, Hattori A (2009) Response of osteoblasts and osteoclasts in regenerating scales to gravity loading. Biol Sci Space 23: 211-217

Suzuki N, Danks JA, Maruyama Y, Ikegame M, Sasayama Y, Hattori A, Nakamura M, Tabata MJ, Yamamoto T, Furuya R, Saijoh K, Mishima H, Srivastav AK, Furusawa Y, Kondo T, Tabuchi Y, Takasaki I, Chowdhury VS, Hayakawa K, Martin TJ (2011a) Parathyroid hormone 1 (1-34) acts on the scales and involves calcium metabolism in goldfish. Bone 48: 1186-1193

Suzuki N, Yachiguchi K, Hayakawa K, Omori K, Takada K, Tabata JM, Kitamura K, Endo M, Wada S, Srivastav AK, Chowdhury VS, Oshima Y, Hattori A (2011b) Effects of inorganic mercury on osteoclasts and osteoblasts of the goldfish scales *in vitro*. J Fac Agr Kyushu Univ 56: 47-51

Taga R, Tang N, Hattori T, Tamura K, Sakai S, Toriba A, Kizu R, Hayakawa K (2005) Direct-acting mutagenicity of extracts of coal burning-derived particulates and contribution of nitropolycyclic aromatic hydrocarbons. Mutat Res 581: 91-95

Thamamongood TA, Furuya R, Fukuba S, Nakamura M, Suzuki N, Hattori A (2012) Expression of osteoblastic and osteoclastic genes during spontaneous regeneration and autotransplantation of goldfish scale: A new tool to study intramembranous bone regeneration. Bone 50: 1240-1249

Tang N, Hattori T, Taga R, Igarashi K, Yang X-Y, Tamura K, Kakimoto H, Mishukov VF, Toriba A, Kizu R, Hayakawa K (2005a) Polycyclic aromatic hydrocarbons and nitro polycyclic aromatic hydrocarbons in urban air particles and their relationship to emission sources in the Pan-Japan sea countries. Atmos Environ 39: 5817-5826

Tang N, Taga R, Hattori T, Toriba A, Kizu R, Hayakawa K (2005b) Simultaneous determination of twenty-one mutagenic nitro polycyclic aromatic hydrocarbons by high-performance liquid chromatography with chemiluminescence detection. Biolumin Chemilumin Prog Perspect World Scientific 441-444.

Tang N, Araki Y, A, Tamura K, Dong L, Zhang X, Liu Q, Ji R, Kameda T, Toriba A, Hayakawa K (2009) Distribution and source of atmospheric polycyclic aromatic hydrocarbons and nitropolycyclic aromatic hydrocarbons in Tieling city, Liaoning province, a typical local city in Northeast China. Asian J Atmos Environ 3: 52-58

Yachiguchi K, Sekiguchi T, Nakano M, Hattori A, Yamamoto M, Kitamura K, Maeda M, Tabuchi Y, Kondo T, Kamauchi H, Nakabayashi H, Srivastav AK, Hayakawa K, Sakamoto T and Suzuki N (2014a) Effect of inorganic mercury and methylmercury on osteoclasts and osteoblasts in the scales of the marine teleost as a model system of bone. Zool Sci 31: 330-337

Yachiguchi K, Matsumoto N, Haga Y, Suzuki M, Matsumura C, Tsurukawa M, Okuno T, Nakano T, Kawabe K, Kitamura K, Toriba A, Hayakawa K, Chowdhury VS, Endo M, Chiba A, Sekiguchi T, Nakano M, Tabuchi Y, Kondo T, Wada S, Mishima H, Hattori A, Suzuki N (2014b) Polychlorinated biphenyl (118) activates osteoclasts and induces bone resorption in goldfish. Env Sci Poll Res 21: 6365–6372

Yoshikubo H, Suzuki N, Takemura K, Hoso M, Yashima S, Iwamuro S, Takagi Y, Tabata MJ, Hattori A (2005) Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. Life Sci 76: 2699-2709

Vaes G (1988) Cellular biology and biochemical mechanism of bone resorption: A review of recent developments on the formation, activation, and mode of action of osteoclasts. Clin Orthop Relat Res 231: 239-271

Zylberberg L, Bonaventure J, Cohen-Solal L, Hartmann DJ, Bereiter-Hahn J (1992) Organization and characterization of fibrillar collagens in fish scales in situ and in vitro. J Cell Sci 103: 273-285

FIGURE LEGENDS

Fig. 1. Locations of sampling sites

Point 1: The Alexandria site on the Mediterranean Sea; Point 2: the Suez Canal site on the Red Sea

Fig. 2. Effect of polluted seawater on ALP activity in cultured scales incubated for 6 hrs

* and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values in the control scales. n = 10 samples; one sample from one fish.

Fig. 3. Effect of polluted seawater on TRAP activity in cultured scales incubated for 6 hrs

There is no significant difference between experimental and control scales at least present conditions. n = 10 samples; one sample from one fish.

Fig. 4. Expression analysis of osteoblastic markers: ALP (A), Osteocalcin (B), and RANKL (C) mRNAs in scales treated with polluted seawater incubated for 6 hrs. ALP, Osteocalcin, and RANKL mRNA levels were normalized to the EF-1α mRNA level. The value of the ordinates indicates the relative ratios of ALP/EF-1α (A), Osteocalcin/ EF-1α (B), and RANKL/ EF-1α (C), respectively. * indicates a statistically significant difference at $P < 0.05$ from the values in the control scales. n = 8 samples; one sample from one fish.

Fig. 5. Expression analysis of osteoclastic markers: Cathepsin K (A), Vacuole-ATPase (B), and Matrix Metalloproteinase-9 (MMP-9) (C) mRNAs in scales treated with polluted seawater incubated for 6 hrs. Cathepsin K, Vacuole-ATPase, and MMP-9 mRNA levels were normalized to the EF-1α mRNA level. The value of the ordinates indicates the relative ratios of Cathepsin K/EF-1α (A), Vacuole-ATPase/ EF-1α (B), and MMP-9/ EF-1α (C), respectively. n = 8 samples; one sample from one fish.

TABLE CAPTION

Table 1 PAH and NPAH concentrations in the seawater of both Alexanderia and Suez Canal sites

Table 2 Effect of PAHs (6 ng/l) and NPAHs (40 pg/l) on ALP activity in cultured scales incubated for 6 hrs